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Evaluation of the reverse transcription-polymerase chain reaction/probe test of serum samples and immunohistochemistry of skin sections for detection of acute bovine viral diarrhea infections

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Abstract. Bovine viral diarrhea viruses (BVDV) cause both acute and persistent infections. While diagnostic tests have been designed to detect animals persistently infected (PI) with BVDV, the reliability of these tests in detecting acute BVDV infections is not known. It is also possible that acute BVDV infections may be confused with persistent infections in surveys for PI animals. In this study, 2 tests presently in use in diagnostic laboratories to test for PI animals, polymerase chain reaction amplification followed by probe hybridization (RT-PCR/probe) of serum samples and immunohistochemical detection of viral antigen in skin biopsies (IHC), were evaluated for their ability to detect acute BVDV infections. Sixteen colostrum-deprived, BVDV-free, and BVDV-antibody-free calves were infected with 6 different BVDV strains. Clinical signs, seroconversion, and virus isolation indicated that inoculated animals did replicate virus. Virus could be detected in 19% (3/16) of acutely infected animals by the RT-PCR/probe technique. No acutely infected animals were positive by IHC.

Bovine viral diarrhea virus (BVDV), a pathogen of ruminants from the pestivirus genus of the flavivirus family, causes a number of diverse diseases affecting the reproductive, respiratory, digestive, and immune systems. Acute infections range from subclinical to highly fatal, depending on the viral strain, host immune status, host reproductive status, and the presence of other pathogens (for review see Houe[7]). Infection in utero can result in the birth of persistently infected (PI) animals. The PI animal is born immunotolerant to the viral strain it carries and will shed virus throughout its lifetime.[11] Prevalence is widespread in most cattle-raising countries. The economic impact is significant, with estimated annual losses between $20–57 million per million calvings.[8]

Bovine viral diarrhea virus infections are difficult to diagnose based solely on clinical signs. Clinical manifestations such as reproductive disorders; birth of malformed, weak, and undersized calves; respiratory disease; diarrhea; and increased susceptibility to other pathogens are not unique to BVDV. In addition, diagnosis of BVDV is confounded by the great variation in the rate of spread of BVDV. Duration of outbreaks varies from a few weeks to several months, depending on whether or not a PI animal is present, the virulence of the BVDV strain, and management and housing practices. Direct contact with PI animals is probably the most frequent method of transmission of infection; however, field studies have shown that spread of infection also occurs in the absence of PI animals.[1,12] Mechanisms of infection in the absence of PI animals are not fully understood but may be due to contact with acutely infected animals, contact with other species infected with BVDV, or indirect means of transmission such as contaminated needles and equipment.

Many diagnostic tests have been designed to detect PI animals. In this study, 2 tests presently in use in diagnostic laboratories, reverse transcription-polymerase chain reaction amplification followed by probe hybridization (RT-PCR/probe) of serum samples and immunohistochemical detection of viral antigen in skin samples (IHC), are evaluated for their ability to detect acute BVDV infections.

Materials and methods

Viral isolates. Six genetically and antigenically distinct BVDV were used (Table 1). Four belonged to the BVDV1 genotype (2 each from subgenotypes BVDV1a and BVDV1b) and 2 belonged to the BVDV2 genotype. Five viruses (2360, 1286, 5013, 6229, 1373) were field strains isolated from acute BVDV outbreaks that were characterized as clinically severe in the field (bloody diarrhea, elevated temperatures, erosions in the mouth and soft palate). One virus (RS886) was isolated from a PI calf. All viruses were noncytopathic and none were coisolated with a cytopathic BVDV. All were propagated on bovine turbinate (BT) cells as follows. Subconfluent monolayers of BT cells grown in 25-cm^2 or 75-cm^2 flasks were inoculated with BVDV strains

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Table 1. Changes in circulating lymphocytes and platelets postinfection.

Virus	Genotype	Number of animals	% change in lymphocytes* ± SE	% change in platelets† ± SE
2360	BVDV1a	2	−47.9 ± 10.1	+23.3 ± 28.3
1286	BVDV1a	2	−42.4 ± 3.7	−37.71 ± 14.0
5013	BVDV1b	2	−19.1 ± 14.6	−11.4 ± 26.8
6299	BVDV1b	2	−41.3 ± 8.6	−35.0 ± 23.0
RS886	BVDV2	5	−57.7 ± 5.2	−12.51 ± 7.6
1373	BVDV2	3	−57.8 ± 2.4	−46.6 ± 5.7
Control	no virus	4	−0.9 ± 7.2	+3.9 ± 8.9

* At day 6 postinfection.
† At day 9 postinfection.

at an approximate multiplicity of infection (M.O.I.) of 10. The cultures, including cells and culture media, were harvested by 2 cycles of freezing and thawing at −20 C 5 days postinoculation. All viruses grew to a titer between 1×10^6 to 1×10^7 viruses/ml in tissue culture.

Calves. Colostrum-deprived, 3- to 9-month-old Holstein or Jersey calves were tested free of BVDV virus in buffy coat (BC) samples by virus isolation, free of BVDV antibodies in serum, and free of BVDV antigen in skin samples (ear notches). The number of calves infected with each strain is listed in Table 1.

Animals were inoculated via the nasal route with 5 ml of tissue culture media with a BVDV tissue culture infectious dose (TCID) of 1×10^6 viruses/ml. Temperatures were taken daily. Blood samples for determination of lymphocyte and platelet counts were collected on days 0, 2, 4, 6, 9, 11, and 13 postinoculation. Lymphocyte and platelet counts were determined using a cytometer[a] per manufacturer's directions. Values were compared by the least significant difference (LSD) method ($P < 0.05$).

Ear notches, serum, and buffy coat samples were collected on days 0, 4, 9, and 13. These time points correlated with the first observation of clinical signs (day 4), midpoint of clinical disease (day 9), and recovery (day 13) based on previous studies.[3,16,17] In those studies, it was noted that elevated temperatures were detected by day 4. Lymphocyte and platelet decreases in comparison with control values were both noted on day 9. Basal temperature commonly peaked between days 7 and 9. By day 13, temperatures and lymphocyte and platelet counts had usually returned to the normal range, viral antigen was cleared or greatly reduced in tissue samples, virus was no longer isolated from buffy coat (BC) cells, and frequently a neutralizing antibody response could be detected in serum.

Isolation of virus from buffy coat. Blood samples for BC isolation were collected in the presence of sodium heparin in Becton Dickinson vaccutainer tubes.[b] Blood cells were separated from plasma by centrifugation at $1{,}800 \times g$ for 25 min. The buffy coat layer was removed by aspiration. The resulting BC sample was frozen at −20 C. For inoculation, a 0.5-ml aliquot of BC freeze/thaw lysate was mixed with 0.5 ml of cell culture media (Eagles MEM supplemented with 10% fetal calf serum). This mixture was added to a 25-cm^2, 60–70% confluent flask of Madin Darby bovine kidney cells (MDBK). After rocking at 37 C for 1 hr, the inoculum was removed from the cells and replaced with 5 ml of cell culture media. After 5 days, the cell culture (including media) was frozen at −20 C. After thawing, 1 ml of the resulting lysate was added to a fresh 25-cm^2 flask of MDBK cells. After rocking for 1 hr at 37 C, 4 ml of cell culture media was added. After incubating for 5 days, total RNA was prepared from the culture and tested for BVDV by RT-PCR. Preparation of total RNA and RT-PCR were performed as described previously.[15] Primer sequences used were the same as those published previously.[15]

Detection of virus in serum (RT-PCR/probe). Viral RNA was extracted from 200 μl of serum using Catrimox-14[c] and 2 M LiCl.[14] The RT-PCR was performed in a multiplex format to identify and differentiate BVDV1 and BVDV2 in a single reaction. The multiplex PCR was based on previously published primer sequences for BVDV 1 and 2.[14] The PCR products were differentiated by their respective sizes on ethidium bromide-stained 2% agarose gels. A 196-bp product was amplified from all BVDV, and an 117-bp product amplified was unique to BVDV2. The PCR products were confirmed to be of BVDV origin by hybridization with a BVDV-specific nonradioactive chemiluminescent-labeled[d] probe. The hybridization probe was generated by PCR using the primers P1 (5′-CTCGAGATGCCATGTGGAC-3′, corresponding to nucleotide positions 224–242) and P2 (5′-GCCTCTGCAACACCCTATC-3′, derived from the complementary strand between bases 327 and 346). The resulting 121-bp hybridization probe, internal to the 196-bp first-round product amplified from all BVDV, was designed to enhance the detection limit from 1 ng to approximately 1 pg of target RNA. All 6 isolates used could be detected in infected freeze/thaw tissue culture cell lysates by this method.

Detection of viral antigen in skin (IHC). Ear notches were either snap frozen in isopentane[9] or fixed in formalin.[13] Frozen tissues were sectioned and immunohistochemistry (IHC) performed as described previously[9] using monoclonal antibodies specific for BVDV1 (CA-1, BZ30) or BVDV2 (BZ52) prepared against the E2 viral polypeptide.[4] Fixed tissues were processed and sectioned, and IHC was performed, as described previously,[13] using an antibody (15C5) prepared against the Erns viral polypeptide.[5,6] All 6 isolates used could be detected by monoclonal antibody staining of infected tissue culture cells.

Detection of serum antibodies. Viral neutralizing titers were determined as described previously.[2] Reference viruses used were BVDV2-1373 and BVDV1b-NY-1.

Table 2. Detection of acute BVDV infection.

Virus	Genotype	Day	Virus isolation BC	PCR serum	Frozen ear notch	Fixed ear notch	Seroconversion
2360	BVDV1a	baseline	0/2	0/2	0/2	0/2	
		4	1/2	0/2	0/2	0/2	
		9	1/2	0/2	0/2	0/2	
		13	0/2	0/2	0/2	0/2	
Total positive			2/2	0/2	0/2	0/2	2/2
1286	BVDV1a	0	0/2	0/2	0/2	0/2	
		4	0/2	0/2	0/2	0/2	
		9	2/2	0/2	0/2	0/2	
		13	0/2	0/2	0/2	0/2	
Total positive			2/2	0/2	0/2	0/2	2/2
5013	BVDV1b	0	0/2	0/2	0/2	0/2	
		4	2/2	0/2	0/2	0/2	
		9	0/2	0/2	0/2	0/2	
		13	0/2	0/2	0/2	0/2	
Total positive			2/2	0/2	0/2	0/2	2/2
6229	BVDV1b	0	0/2	0/2	0/2	0/2	
		4	2/2	1/2	0/2	0/2	
		9	0/2	1/2	0/2	0/2	
		13	0/2	0/2	0/2	0/2	
Total positive			2/2	1/2	0/2	0/2	2/2
RS886	BVDV2	0	0/5	0/5	0/5	0/5	
		4	5/5	0/5	0/5	0/5	
		9	5/5	2/5	0/5	0/5	
		13	0/5	1/5	0/5	0/5	
Total positive			5/5	2/5	0/5	0/5	3/5
1373	BVDV2	0	0/3	0/3	0/3	0/3	
		4	1/3	0/3	0/3	0/3	
		9	3/3	0/3	0/3	0/3	
		13	1/3	0/3	0/3	0/3	
Total positive			3/3	0/3	0/3	0/3	3/3
Control	No virus	baseline	0/4	0/4	0/4	0/4	
		4	0/4	0/4	0/4	0/4	
		9	0/4	0/4	0/4	0/4	
		13	0/4	0/4	0/4	0/4	0/4

Results

All infected animals had greater than a 0.5 C rise in temperature for at least 1 day postinfection. In addition, infection with all 6 viruses resulted in a significant decrease in circulating lymphocytes as compared with control values (Table 1). Infection with 1 of the isolates resulted in a significant drop in platelets. Infection with 4 of the other strains averaged a reduction in platelets; however, the small number of animals used and the large within-group variation resulted in failure to detect significant differences from the control values. While clinical signs were seen with all isolates, clinical disease was more severe with the BVDV2 isolates. Virus could be isolated from the buffy coats of all infected animals on at least 1 day postinfection (Table 2). All 8 animals infected with BVDV1a and BVDV1b isolates seroconverted by day 14. Six out of 8 animals infected with BVDV2 isolates seroconverted by day 14. Failure of all 8 BVDV2-infected animals to seroconvert by day 14 may be related to the effects of viral infection. Clinical symptoms of the animals infected with the BVDV2 isolates were more severe. Further, severe depletion in immune tissues has been demonstrated in animals infected with virulent BVDV2 isolates.[17] It is possible that depletion may have slowed seroconversion in severely affected animals.

Variation was observed between strains on how soon and how long virus could be isolated from BC. The window of isolation did not correlate with the peak of clinical signs (largest drop in white blood cells, highest recorded temperature) with some isolates. Infection was not detected in any of the infected animals by either of the IHC techniques. Virus was detected in the serum of 2 of the animals infected with a BVDV2 isolate and 1 animal infected with a BVDV1b isolate by the RT-PCR/probe technique.

Discussion

Virus isolation from BC, seroconversion, and clinical signs indicate that all 6 viruses were replicating in the animals during the testing period. The IHC staining

did not detect virus in skin biopsies (ear notches) of acutely infected animals on any of the testing dates with any of the 6 isolates. A previous report noted that BVDV antigens did not accumulate in skin tissue in acute infections when a low dose (10^5 TCID) of virus was used.[13] However, in that study, all calves tested were negative for BVDV by virus isolation from either serum or buffy coat samples at the time of skin collection. Thus, this previous study did not demonstrate that the animals tested had a systemic viral infection current to the collection of skin samples. When calves were infected with a higher dose (10^8 TCID) of virus, BVDV could be isolated from serum and buffy coat samples. Skin biopsied for BVDV was positive in 40% of these animals. The present study used a viral inoculation dose intermediate to the 2 doses used in that study, isolated buffy coat at time of skin testing, but did not see viral antigen accumulation in skin. On occasion, viral antigen accumulation in skin is observed in severe acute BVDV2 infections, even when lower dosages of the virus are used (E. Liebler-Tenorio, Institute for Pathology, School of Veterinary medicine, Hannover, Germany, personal communication). However, this was only observed in moribund animals. A similar phenomenon is observed with the superinfecting cytopathic virus in mucosal disease. Usually the cytopathic virus replicates in limited locations; however, in some moribund animals, the cytopathic virus is no longer restricted and appears in most tissues, including skin.[10] In summation, this study and previous studies indicate that skin biopsy tests will usually not confuse PI animals with acutely infected animals. However, these methods are unreliable for detection of acute infections.

The RT-PCR/probe technique detected acute infection in 1 of the 8 animals infected with a BVDV1 isolate and in 2 of the 8 animals infected with virulent BVDV2 isolates. Previous research has revealed that the average maximal titer of virus detected in serum is strain dependent.[3] A 1,000-fold difference was seen between the maximal titers of different BVDV isolates. Detection of some isolates but not others may be a function of variation of viral serum titer. However, the number of animals observed in this study was not adequate to make those comparisons. Regardless, most of the acutely infected animals, 13/16 (81%), were not detected by this method. Detection of acute infections with this technique might be improved by using BC samples rather than serum.

These results suggest that IHC of skin biopsies and the RT-PCR/probe test, which are now used to screen herds for PI animals, will not reliably detect acute outbreaks of BVDV. Inasmuch as detection of acute infections is as important as detection of PIs to biosecurity, one should not be lulled into complacency by tests that are designed to detect PI. Failure to detect persistently infected animals does not mean that BVDV is not a herd health problem. However, screening for acute BVDV infections in herds can be problematic. While virus isolation from BC is reliable, it is time consuming and expensive. Further, the time frame in which virus can be isolated from BC is narrow. The result is that acute BVD infections are most likely underreported and the effects of BVDV on the general health of national herds is underestimated.

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Sources and manufacturers

a. CDC Technologies, Inc., Oxford, CT.
b. BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ.
c. Iowa Biotechnology Corp., Coralville, IA.
d. Mermaid kit, Bio 101, La Jolla, CA.

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